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14. ABSTRACT

The aim of this study was to demonstrate, in guinea-pigs in vivo, the utility of huBuChE as a therapy against percutaneous (p.c.) nerve agent poisoning, following which there is a slower absorption of agent than by the inhalation route and consequently a longer "window of opportunity" for therapeutic intervention. The first task was to determine the p.c. toxicity of VR in Dunkin-Hartley guinea-pigs. The 24h LD50 was 0.45mg/kg (0.36 – 0.54 95% CI) and the 48-h LD50 was 0.38 mg/kg (0.31-0.45 95% CI). The second task was to investigate the efficacy of huBuChE therapy following p.c. VR. Following VR (0.6mg/kg, p.c.) guinea-pigs were monitored for the onset of observable signs of systemic cholinergic poisoning. At this time, either huBuChE (24.2 mg/kg) or saline was administered by either the intramuscular or intravenous route. The onset of observable signs of cholinergic therapy used as the trigger for therapy dosing was between 5h and 9h following VR challenge. Preliminary results indicate that therapy using huBuChE via the i.v. route was more effective than therapy via the i.m. route.

15. SUBJECT TERMS
Bioscavenger; nerve agent; percutaneous; guinea-pig, VR, medical countermeasures

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Introduction

Human butyrylcholinesterase (huBuChE) has investigational new drug (IND) status in the U.S. as a pretreatment against organophosphate poisoning in humans, and huBuChE pretreatment provides significant protection against the lethal effects of nerve agents in animals. Less is known about the efficacy of huBuChE against percutaneous nerve agent challenge. The aim of this study was to determine, in vivo, the utility of huBuChE as a therapy against percutaneous nerve agent poisoning, following which there is a slower absorption of agent than by the inhalation route and consequently a longer "window of opportunity" for therapeutic intervention. The work in this project will characterize the extent of the beneficial effects of huBuChE, in terms of efficacy and window of opportunity for therapeutic intervention, following percutaneous challenge with VR. The work program was divided into two sub-tasks:

1. Conduct experiments to determine the LD_{50} of percutaneously-applied VR in guinea-pigs.
2. Conduct experiments to investigate the efficacy of huBuChE therapy following percutaneous VR. Use telemetric monitoring to determine the effects of poisoning and huBuChE on selected physiological indicators.

Body

All experiments were conducted according to the terms and conditions of project licences issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

Task 1

Task 1 Aim: To determine the LD_{50} of percutaneously-applied VR in guinea-pigs.

Task 1 Methods:

VR was synthesised by Detection Department, Dstl, Porton Down at 99% purity and supplied in isopropyl alcohol at approximately 30mg/ml.

Male Dunkin-Hartley guinea-pigs (Harlan, UK) were implanted on arrival with a temperature/identity transponder and singly housed. Body weight and temperature were recorded daily throughout the experiment. The guinea-pigs were kept in standardised conditions throughout the study, according to U.K. Home Office guidelines (room temperature 21°C, humidity 50%). The lights were on from 06:00 to 18:00.

Historical VX data were used to identify the range of starting doses for VR. The experiment commenced with the chosen dose challenges. Interim analysis was undertaken after 12 animals, and an adaptive design was used to select additional doses to improve the precision of the probit analysis for the final six animals (Stallard, 2006; Russell et al, 2009). Animals were dosed in groups of 3, and on each experimental day a separate aliquot of stock VR was diluted to the appropriate concentrations in isopropyl alcohol and applied to a clipped area of the dorsal skin at a dose volume of 0.033 ml/kg. VR dosing was carried out in a fume cupboard in which the animals remained for 7h under continuous observation. Guinea-pigs were then returned to a laboratory and observed periodically thereafter. Clinical signs of VR poisoning e.g. tremor, incapacitation, lachrymation and fasciculations were scored during the observation periods. Incapacitation was classified as normal, mild, moderate or substantial (Wetherell et al, 2002). Survival was determined at 24h and 48h. A post-mortem examination was carried out on animals that died during the study and on animals euthanized at the end of the experiment at 7 days.

The Probit regression model was applied to the experimental data using a Generalized Linear Model in the R Statistical Software package (http://www.r-project.org/). The LD_{50} metric was calculated from the Probit regression equation by rearranging the model formula in terms of dosage. The Probit model equation is:

$$\phi(p) = \beta_0 + \beta_1 Dose$$

The $\phi(p)$ is the Probit link function so this formula can be used more generally for calculating LD_p metrics rather than just the LD_{50}.

The formula to calculate the Dose required for a given probability, p, of death is shown here:

$$Dose = \frac{\phi(p) - \beta_0}{\beta_1}$$

Approximate standard errors for this dose estimate were calculated using the transformation method and confidence intervals using the Delta method.

Task 1 Results:

The toxicity determination used a total of 18 male guinea-pigs. On the day of dosing the average body weight was 387 ± 9g, (SD). The Probit Regression model was fitted to all the data on completion of the study. Although the trials were designed for the 24 hour outcome, the 48 hour data were also well described by the model.

The outcome provided an accurate estimate of the main metric of interest, the LD_{50}, but additionally the LD_{95} and LD_{99} have been estimated with greater precision than was expected. The results are summarised in the tables below.

			95% Confidence Interval	
Prob Es	**timate (mg/kg)**	**Standard Error**	**Lower**	**Upper**
LD_{50} 0	.45	0.04	0.36	0.54
LD_{95} 0	.67	0.10	0.47	0.87
LD_{99} 0	.76	0.14	0.49	1.03

Table 1. LD_{50}, LD_{95} and LD_{99} estimates at 24 hours following percutaneous challenge with VR in the guinea-pig.

			95% Confidence Interval	
Prob Es	**timate (mg/kg)**	**Standard Error**	**Lower**	**Upper**
LD_{50} 0.38		0.04	0.31	0.45
LD_{95} 0.52		0.06	0.40	0.64
LD_{99} 0.58		0.08	0.41	0.74

Table 2. LD_{50}, LD_{95} and LD_{99} estimates at 48 hours following percutaneous challenge with VR in the guinea-pig.

These data were used to choose a challenge dose for the next study task. A suitable challenge dose would result in a high probability of animals dying within 48h in the absence of effective medical countermeasures. The chosen dose was 0.6 mg/kg. This represents 1.3 × the 24h LD_{50} of 0.45mg/kg and is approximately equal to the 48h LD_{99}.

Task 2

Task 2 Aim: To investigate the efficacy of huBuChE therapy following percutaneous VR.

Task 2 Methods:

Body weight and temperature were recorded daily throughout the experiment. The guinea-pigs were kept in standardised conditions throughout the study, according to Home Office guidelines (room temperature 21°C, humidity 50%). The lights were on from 06:00 to 18:00.

Surgery: Animals were surgically prepared according to procedures previously published (Mumford et al, 2010). Briefly, 14 days prior to VX challenge, male Dunkin Hartley guinea-pigs (Harlan Interfauna) were implanted under isoflurane anaesthesia (1.5-2% with O_2 0.8 l/min and N_2O 0.8 l/min) with telemetry transmitters capable of measuring body temperature, locomotor activity, ECG and EEG (TL11M2-F40-EET, Data Sciences International, St Paul, USA). Animals were allowed to recover for 14 days prior to nerve agent dosing.

In order to administer therapy via the intravenous route, some animals underwent a second procedure 48h prior to VR challenge, to implant a jugular venous cannula under halothane anaesthesia.

Experimental design: The study design is shown in the table below.

Time of therapy	Therapy (route) huBuChE	Control (route) Saline	Number of animals planned
1. Observed signs	8 (i.m.)	8 (i.m.)	16
2. Observed signs	8 (i.v.)	8 (i.v.)	16
3. Shorter time	8 (i.m.)	8 (i.m.)	16

Table 3 Planned dose groups in Task 2 of the study.

A group size of 8 was chosen based on a Log Rank Test Power Analysis requiring a proportional difference of 0.6 in the number of animals surviving between the control and treated groups, to achieve statistical significance for a power of 70%. Animals were usually dosed in pairs and randomly assigned to receive either therapy or control treatment.

Nerve agent dosing: On the day of dosing the average body weight was 470 ± 32g, (SD). An area of skin on the back was close-clipped. Eighteen hours later VR (18.2 mg/ml) was applied to the prepared area of skin at a dose volume of 0.033 ml/kg giving a dose of 0.6 mg/kg.

HuBuChE (Baxter) was purified from out-dated blood under conditions of Good Manufacturing Practices (Kolarich et al, 2002), and was provided in saline at 24.2 mg/ml (619 units/mg). Saline vehicle (1ml/kg) or huBuChE (24.2 mg/kg) was administered (i.m.) in equal volumes in each of the thigh muscles of the hind legs or i.v. using a jugular venous cannula.

The assessment of signs of poisoning as a trigger for therapy administration was made on an individual animal basis, being unequivocal evidence of systemic cholinergic poisoning. The criteria for huBuChE dosing were mild or moderate incapacitation (Wetherell et al, 2002) accompanied by at least two of the following signs: visible salivation, lachrymation or severe tremor. The onset of seizure activity was not itself a trigger, but if present was typically accompanied by a rapid deterioration in observable condition.

VR dosing was carried out in a fume cupboard. A 90-minute pre-dosing period was allowed for acclimatization. Following VR dosing, the animals remained in the fume cupboard for 7h under continuous observation. They were then returned to their usual laboratory and observed periodically thereafter. A post-mortem examination was carried

out on animals that died during the study and on animals euthanized by anaesthetic overdose at the end of the experiment at 7 days. Tissue samples (plasma, erythrocytes and selected brain areas) were retained for cholinesterase activity determination.

Cholinesterase analysis: Results are not yet available for cholinesterase determination. Previously-published brain area cholinesterase activity data from naïve weight-matched control animals are available for comparative purposes (Mumford et al, 2008).

Data analysis: Animals were weighed daily, and post-surgery the weight of the transmitter (8g) was taken into account. Telemetry data were collected using commercial software (Dataquest ATR V4.2, Data Sciences International, USA) with segment duration 60 sec. ECG and EEG were recorded at sampling frequencies of 500 Hz and 250 Hz respectively. Heart rates were derived from the ECG waveform by the computer software and expressed as mean beats per minute for each minute of data. Temperature was measured through a sensor in the implant body and recorded every minute. Continuous telemetry recording was begun at least 24h prior to VR dosing. Control heart rate and temperature were taken as the mean of the last 30-minute predosing period for each individual animal. Bradycardia (reduced heart rate) was defined for the purposes of this study as a 25% fall from the 30-minute predosing average, sustained for at least 3 consecutive minutes of valid data. The time of first occurrence of bradycardia following VR was determined on an individual animal basis. Seizure was determined by visual examination of the EEG trace, either concurrently with observations of animal behaviour or retrospectively by viewing the saved waveforms, and characterised by regular high-amplitude spike activity, either continuous or in intermittent bursts, each of a few seconds duration.

Data are presented as mean ± standard deviation unless otherwise stated. Statistical comparisons were performed using GraphPad Prism (GraphPad Software, Inc., USA).

Task 2 Results:

To March 2011, 34 animals underwent surgical procedures to implant telemetry transmitters. A total of 30 animals have been successfully dosed in the study. All 16 animals in group A (Table 3) have been completed, and 14 out of 16 in Group B. The remaining 4 animals did not meet the criteria to progress to dosing, either as a result of failure to attain an appropriate dosing weight or because of infection resulting from the surgery.

The survival following VR and therapy is shown in Figure 1. No statistical analysis is available as the data are incomplete. Five out of 15 saline-treated animals survived to 7 days; 9 died between 9 and 38h as a result of VR poisoning and one was euthanized at 48h on grounds of excessive weight loss and continuing signs of poisoning. All animals lost approximately 10% of predosing bodyweight at 24h (Figure 2).

When huBuChE was administered by the i.m. route on signs of poisoning, 3/8 animals died between 10 and 29h as a result of VR poisoning; a further 2 were euthanized (at 48h and 72h) due to excessive weight loss. Three animals survived to the end of the experiment at 7 days. The i.v. route of huBuChE administration would be expected to improve the outcome following p.c. poisoning due to the faster time to achieve peak plasma levels and the higher bioavailability. The results obtained so far have confirmed this, with 6/7 animals surviving to 7 days. One animal was euthanized at 48h due to excessive weight loss and continuing signs of poisoning. The surviving animals regained their pre-dosing weight by Day 5 (Figure 2).

The time to onset of signs of poisoning, and hence dosing time, was variable, as expected following poisoning by this route. There appeared to be no difference in mean dosing times between dose groups (Figure 3, Table 3).

Further analysis of physiological data on an individual animal basis is ongoing. Assessment criteria contributing include survival; incidence and timing of bradycardia, seizure and hypothermia; post-mortem assessment of gross visual changes in thoracic and abdominal organs; and cholinesterase inhibition in blood and selected brain areas.

Prior to the commencement of the final dose groups (Table 3, Group C) an assessment will be made of the appropriate therapy time to be used in that group.

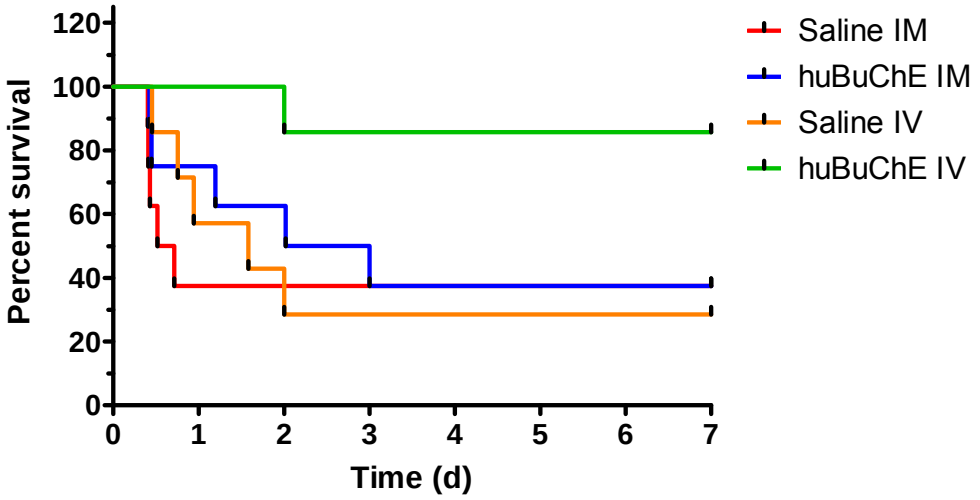

Figure 1 Survival following VR and therapy on signs of poisoning as shown in key. Interim results: n=8 for i.m. groups and n=7 for i.v. groups.

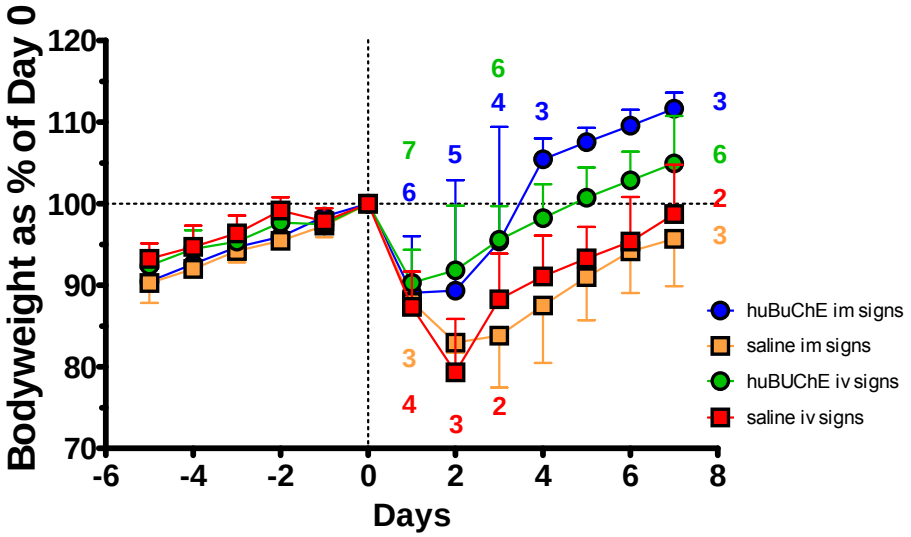

Figure 2 Bodyweight (relative to day of dosing) of surviving animals following agent challenge and therapy on signs of poisoning as shown in key. Interim results: n=8 for i.m. groups and n=7 for i.v. groups at the time of dosing; subsequent group sizes are shown on the graph to denote the times at which animals died or were euthanized.

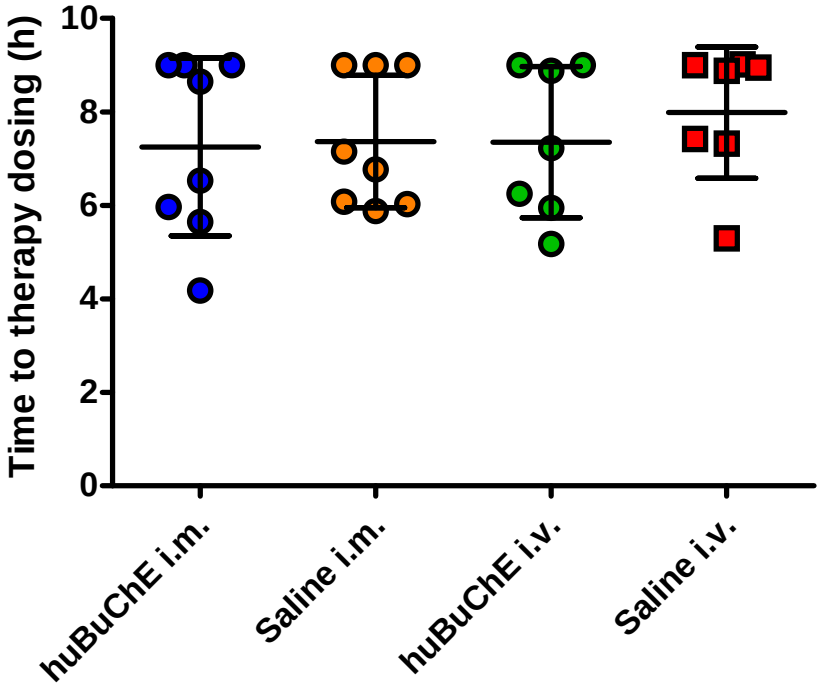

Figure 3 Time of therapy dosing on signs of poisoning. Interim results: n=8 for i.m. groups and n=7 for i.v. groups. Error bars are mean +/- SD.

Dose Group	Number dosed	Time of dosing (h) (mean ± SD)	Survival 48h	Survival 7 days	Bradycardia incidence	Abnormal lungs at post mortem	Abnormal GI tract at post mortem
huBuChE i.m. (signs of poisoning)	8	7.25 ± 1.90	5/8	3/8	5/8	7/8	1/8
huBuChE i.v. (signs of poisoning)	7	7.36 ± 1.62	7/7	6/7	3/7	4/7	0/7
Saline (i.m.) (signs of poisoning)	8	7.36 ± 1.42	3/8	3/8	7/8	7/8	2/8
Saline (i.v.) (signs of poisoning)	7	7.98 ± 1.40	3/7	2/7	6/7	5/7	3/7

Table 4 Summary of animals dosed so far with survival outcomes and post-mortem findings. Animals were dosed via the percutaneous route with VR (0.6 mg/kg) and either huBuChE (24.2 mg/kg) or saline (1ml/kg) was administered on observable signs of systemic cholinergic poisoning (see methods).

Key Research Accomplishments

The percutaneous toxicity of VR was determined to have a 24h LD_{50} of 0.45mg/kg (0.36 – 0.54 95% CI) and a 48-h LD_{50} of 0.38 mg/kg (0.31-0.45 95% CI).

There is a window following p.c. VR poisoning in which post-exposure therapy with huBuChE has some efficacy when administered via the i.m. route at the onset of signs. As expected, interim results suggest that the outcome was improved when therapy was administered via the i.v. route. Full analysis will take place when all animals in the groups have been dosed. Interim analysis suggests that:

- The challenge dose chosen (0.6 mg/kg, or 1.3 × LD_{50}) produced the expected range of effects and onset times.
- A higher than expected proportion of saline-treated animals survived the VR challenge. This result reflects the uncertainty in the derived LD_{50} values, and the inherent variability of the p.c. dosing route.
- The onset of observable signs of cholinergic therapy used as the trigger for therapy dosing was between 5h and 9h following VR challenge.
- Therapy using huBuChE via the i.v. route was more effective than therapy via the i.m. route.

Reportable Outcomes

No reportable outcomes so far.

Conclusions

This study has shown that, following percutaneous nerve agent poisoning, post-exposure therapy using huBuChE has potential as a treatment strategy. This finding complements recently published studies demonstrating the efficacy of huBuChE and recombinant butyrylcholinesterase following percutaneous VX poisoning in guinea-pigs and swine (Mumford et al, 2011, Lenz et al, 2010, Tenn et al, 2008). We have shown that there is a limited therapeutic window in case of pc VR poisoning and the protective effect of huBuChE is not substantial when administered via the i.m. route at the onset of signs. As expected, interim results suggest that the outcome was improved when therapy was administered via the i.v. route. Previous studies have demonstrated that bioscavenger, administered prior to the onset of observable cholinergic signs of VX poisoning, afforded almost complete protection (Mumford et al, 2011). The next phase of the current work will investigate this scenario for p.c. VR challenge. Full conclusions may not be drawn from these data as the studies are not yet complete and all the data are not yet available. The opportunity for post-exposure treatment may have particular relevance in civilian settings, and this is a promising indication for the use of huBuChE.

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Appendix – Financial Summary

QUARTERLY FINANCE UPDATE

1. Contract No **W81WXH-10-C-0044**

2. Reporting period from 1st January to 31st March

3. Current staff, with percent effort of each on project (Jan-Mar 2011).

Staff/Role %	**Effort This Qtr**
Helen Mumford (PI)	38.6
L3 Scientist	57.0
L4 Scientist	24.9
L5 Scientist	0.0
Animal Welfare Staff	4.6
Statistician 0	.0
Lead Technical Reviewer	1.1
Project Manager	7.6

10. Comments on administrative and logistical matters.

Contract modification currently under review; Modification No: P00001 'pd2AB' Contract No: W81XWH-10-C-0044